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VideoHacking: Automated Tracking and Quantification of Locomotor Behavior with Open Source Software and Off-the-Shelf Video Equipment

Emily E. Conklin, Kathyann L. Lee, Sadie A. Schlabach, Ian G. Woods
Department of Biology, Ithaca College, Ithaca, NY 14850

Differences in nervous system function can result in differences in behavioral output. Measurements of animal locomotion enable the quantification of these differences. Automated tracking of animal movement is less labor-intensive and bias-prone than direct observation, and allows for simultaneous analysis of multiple animals, high spatial and temporal resolution, and data collection over extended periods of time. Here, we present a new video-tracking system built on Python-based software that is free, open source, and cross-platform, and that can analyze video input from widely available video capture devices such as smartphone cameras and webcams. We validated this software through four tests on a variety of animal species, including larval and adult zebrafish (*Danio rerio*), Siberian dwarf hamsters (*Phodopus sungorus*), and wild birds. These tests highlight the capacity of our software for long-term data acquisition, parallel analysis of multiple animals, and application to animal species of different sizes and movement patterns. We applied the software to an analysis of the effects of ethanol on thigmotaxis (wall-hugging) behavior on adult zebrafish, and found that acute ethanol treatment decreased thigmotaxis behaviors without affecting overall amounts of motion. The open source nature of our software enables flexibility, customization, and scalability in behavioral analyses. Moreover, our system presents a free alternative to commercial video-tracking systems and is thus broadly applicable to a wide variety of educational settings and research programs.

Key words: video tracking; zebrafish; ethanol; thigmotaxis; open source; Python

The quantification of behavior is a powerful, non-invasive method for studying nervous system function, as differences in behavior arise from differences in neural activity. For example, locomotor output is a robustly quantifiable behavior that is sensitive to genetic and pharmacological perturbations of nervous system activity and to differences in sensory input (Rothenfluh and Heberlein, 2002; Lebestky et al., 2009; Rihel et al., 2010; Swierczek et al., 2011; Woods et al., 2014). Locomotion can be quantified via direct observation, but these assays are time- and labor-intensive, require extensive training, and can be confounded by subjective differences between observers, and drift due to observer fatigue. Automated assays of animal movement enable simultaneous tracking of multiple animals and objective quantification of locomotor behaviors. Furthermore, video-based analyses enable high sampling frequency, high spatial resolution, and long periods of data collection. These characteristics eliminate potential observer biases and enable quantification of behaviors that might be missed via manual observation, such as rapid movements, rare behaviors, or patterns of locomotion that emerge over extended periods of time (Noldus et al., 2001).

Though commercial systems exist for video tracking of locomotor behaviors, the significant costs of the hardware and software used in these systems limit their broad applicability. In addition, customization of these systems often requires hardware modifications and additional software that can increase costs further. To overcome these limitations, video tracking tools have been developed that use freely available software and inexpensive video equipment (Togasaki et al., 2005; Ramazani et al., 2007; Aguiar et al., 2007). Similarly, technologies already in widespread use have been creatively repurposed to quantify animal locomotion. For example, Pittman and Ishikawa (2013) employed smartphone applications originally designed for slow-motion analysis of golf club swings and other sports movements to quantify locomotor behaviors in zebrafish.

Here, we have developed video tracking software to quantify locomotor behaviors, based on the widely available programming language Python and the OpenCV set of open source computer vision tools. Our system is designed to process and analyze video acquired from commonly available image capture devices, including smartphones, laptop computers, and inexpensive video cameras. Briefly, our system (1) allows users to delineate regions of interest in a field of view, (2) quantifies locomotion in each region of interest by calculating frame-to-frame pixel changes in the video feed, (3) allows the user to group regions of interest according to treatment or genotype if appropriate, and (4) displays the results of the experiment in a variety of user-specified ways. Our video tracking system thus facilitates quantification of locomotor behavior for any organism, is flexible in terms of video input, is based on free and cross-platform software, and can be run on mobile devices and personal computers using a variety of operating systems. This flexibility will enable widespread implementation in undergraduate neuroscience and animal behavior labs, and will facilitate rapid and inexpensive prototyping of behavioral assays.

MATERIALS AND METHODS

Experimental setup. Because motion is detected by quantifying pixel differences between successive video frames, care must be taken to eliminate potential sources of motion artifacts, such as moving shadows, glare,

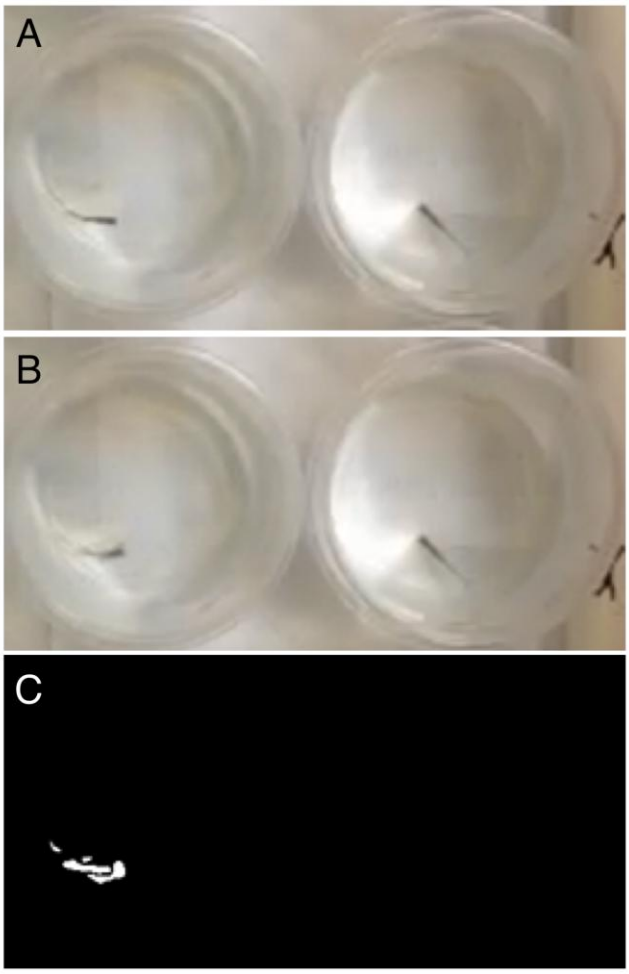

Figure 1. Quantification of movements via pixel differences. *A, B.* Frames from a movie acquired on a smartphone showing two adult zebrafish in circular plastic containers. Color movies are converted to grayscale and blurred slightly to reduce background motion artifacts. Each movie frame is then represented mathematically by an array of numbers representing pixel intensities. *C.* Subtraction of successive video frames highlights regions that are different between the two frames and represent movement (white), while background regions and stationary animals are omitted (black), enabling quantification of the timing and magnitude of movements.

changes in light intensity, or warping in the image resulting from lens curvature. Similarly, when analyzing the behavior of multiple animals simultaneously, confounding variables such as enclosure size, age, or sex should be minimized. Though the selection of the regions of interest to analyze is flexible, the software presented here is especially suited for samples arranged in a grid, such as in multi-well cell culture plates or multiple adjacent enclosures of similar size and shape.

Software acquisition and installation. The core computational modules require Python and the Python libraries matplotlib (www.matplotlib.org) and NumPy (www.numpy.org). These are included as part of the current OS X operating system, and can be easily installed on computers using Windows or Linux. The image analysis components of the system require OpenCV (www.opencv.org), which is compatible with all operating systems in common use. The optional graphical user interface requires installation of PyQt4 (www.riverbankcomputing.com/software/pyqt/download). Instructions for use and details regarding how the software functions are available as part of the software package download at http://faculty.ithaca.edu/iwoods/docs/.

Software operation. The video tracking software is based upon three core modules, as outlined below. These modules can be accessed via the command line interface, or via the Python-based graphical user interface included in the software package.

roiSelect.py. This module enables demarcation of regions of interest (ROIs) by retrieving the first frame of a video file or from the live video feed of an attached or built-in camera if no file is specified. The video frame is displayed on screen, and the user may select ROIs on the image by drawing rectangles of any size. If only one rectangle is drawn, the user is prompted to partition the large rectangle into smaller ROIs by entering the desired number of rows and columns. This partitioning step enables the generation of gridded ROIs, which are useful for animals arrayed in a regular pattern. Each region of interest is numbered by position and is assigned a corresponding color label.

deltaPix.py. This module quantifies motion in the video feed by reading the video stream frame-by-frame and calculating the differences in pixels between adjoining frames. The resulting difference image highlights where motion has occurred between the two frames. In this manner, both the background and non-moving animals appear black, whereas moving objects appear white (Figure 1). The amount of movement in each ROI is recorded as the number of pixels that are different in the two subsequent frames, and the time at which this difference occurs is also recorded. Thus, both the magnitude and timing of each movement are quantified and saved in a matrix, with the rows corresponding to pixel differences for each frame, and the columns corresponding to each ROI, with an additional column for time.

analyzeMotion.py. This is the data analysis module, which can display the results of deltaPix.py in a variety of ways as specified by the user (see Figures 2-5 for examples). Results can be displayed for each individual ROI, or for groupings of ROIs according to differences in treatment or genotype. Data display is customizable for each experiment, and with additional coding can be applied to more complex analyses of movements at high temporal resolution (Woods et al., 2014).

Ethanol treatment of adult zebrafish. Two hours before data collection, male zebrafish at six months of age were placed into 2-liter tanks at a density of six fish per tank. One hour before data collection, 10 mL of ethanol was added to the treatment tanks (for a final concentration of 0.5% vol/vol), and 10 mL of system water was added to the control tanks. Five minutes before data collection, the fish were transferred to a rounded square plastic container (23 cm x 23 cm x 7 cm, 3.07 L volume, GLAD) containing 2 L

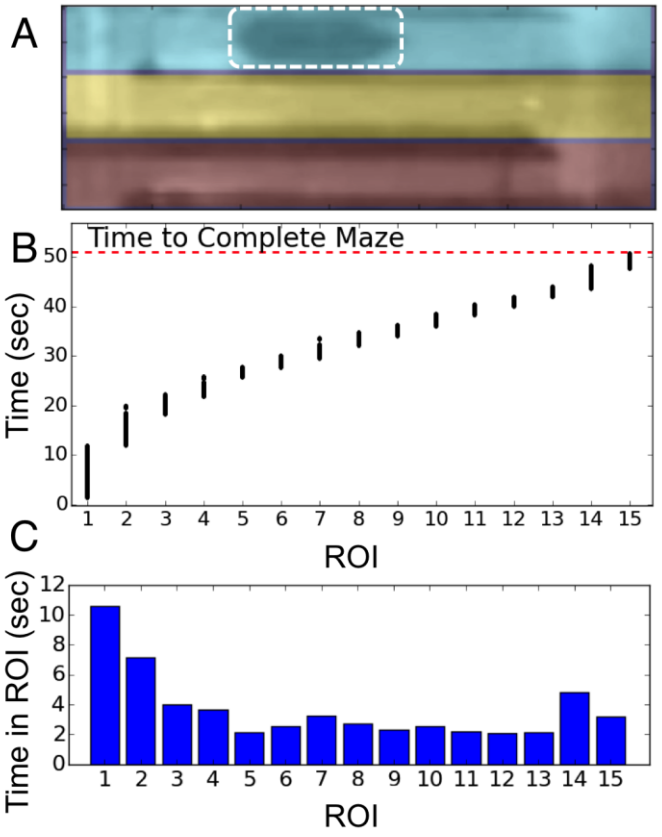

Figure 2. Quantification of locomotion in a prerecorded movie. *A.* A frame of a movie showing three rows of a simple 15-row maze. The circled region (dotted white line) denotes a Siberian dwarf hamster navigating one of the rows of this maze. *B.* Timing of movement through the rows of the maze. Each frame that contained movement was represented by a dot on the plot; continuous movements were thus represented as lines. *C.* Quantification of motion in each row of the maze. The bars show the time spent by the hamster in each row. After a slow start (~11 seconds in the first row, ~7 seconds in the second row), the hamster moved relatively quickly through each row (~2-3 seconds/row) and completed the maze in about 51 seconds.

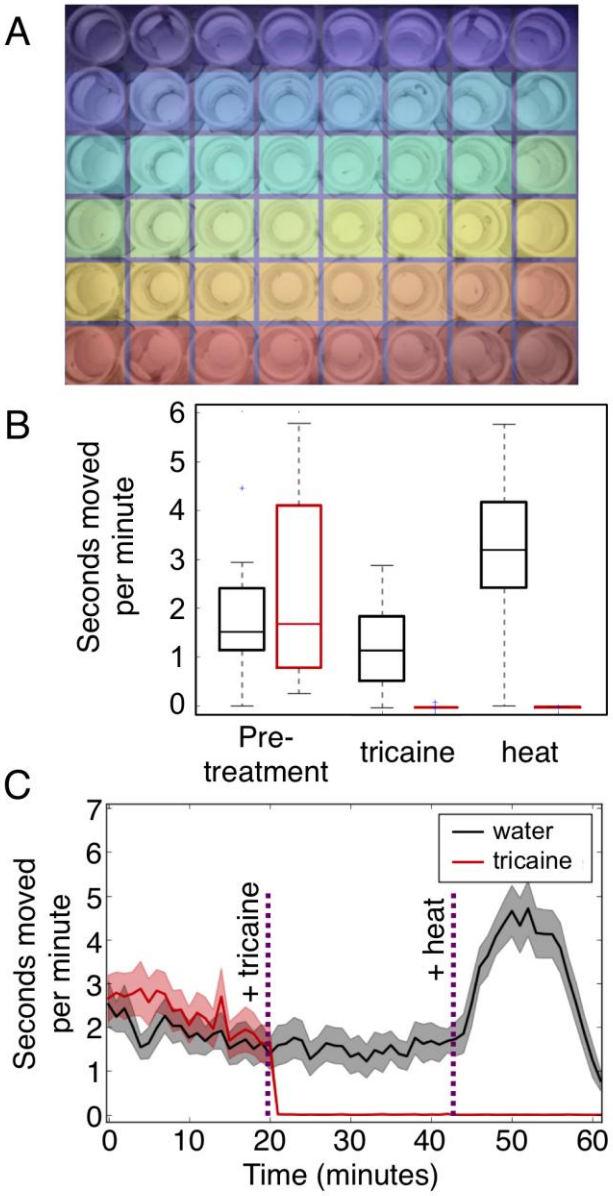

Figure 3. Simultaneous tracking of 48 larval zebrafish with a smartphone. *A.* Regions of interest in the multi-well plate. The image is a frame from a movie taken with a smartphone, and the different colors represent each region of interest (ROI). The arrayed ROIs are generated automatically, by partitioning a rectangle drawn over the entire plate into a grid based on user-entered numbers of rows and columns. *B.* Quantification of movement differences. In the twenty minutes prior to treatment with tricaine, movements of control and experimental larvae were statistically indistinguishable ($p = 0.4$; n=24 for each condition). Larvae treated with tricaine became largely immotile compared to their control siblings ($p < 0.0001$). Upon exposure to warm water, control larvae exhibited increased locomotion ($p < 0.001$ comparing controls before and after heat exposure), whereas tricaine-treated fish remained immotile ($p < 0.0001$ comparing tricaine-treated fish with controls after heat exposure). All statistical comparisons are via two-tailed t-tests. *C.* Overview of locomotion. The average locomotion of 24 tricaine-treated larvae (red) and 24 untreated siblings (black) is shown by the dark lines, while the shaded regions represent +/- s.e.m.

of system water. Locomotor behavior was recorded with a Panasonic HX-WA03 video camera affixed to a tripod. An umbrella was fastened to the top of the tripod to minimize glare from overhead lights. Data were collected between 10 am and 2 pm. Experiments were performed on fish from the TL strain, and on fish from a TL x AB cross; results from both strains were similar.

RESULTS AND DISCUSSION

The video tracking software described here can be applied to a wide range of animal species, and can be customized to fit a variety of educational programs and experimental interests. Sample experiments are described below to demonstrate the flexibility of data acquisition and visualization provided by the software.

Timing of maze navigation. To test the ability of our program to quantify animal locomotion from pre-recorded

videos, we analyzed a movie of a single Siberian dwarf hamster navigating through a simple maze. The fifteen rows of the maze were partitioned by roiSelect.py into rectangular regions of interest (ROIs) of equal size (Figure 2*A*). Motion in each row was recorded by deltaPix.py, and analyzeMotion.py was used to visualize data in two different ways. First, the timing of motion in each ROI (i.e., each row of the maze) was represented by plotting a dot whenever a pixel difference was detected in that ROI (Figure 2*B*). Second, the total time spent in each ROI was plotted in a bar graph (Figure 2*C*). The hamster spent a longer period of time navigating through the first two rows of the maze. By analyzing multiple videos of this type, the average time spent in each row or the time to complete the entire maze can be objectively calculated and compared between animals of different genotypes or different treatment regimes. Thus, our video tracking program is capable of automated, rapid, bias-free analysis of animal motion at high temporal resolution, and visualizing the data in ways meaningful to the user.

Response of larval zebrafish to tricaine and heat. To test the ability of our video tracking system to process video acquired from a smartphone, and to assess motion detection of a large number of small animals simultaneously, we quantified the effects of an anesthetic and heat on larval zebrafish (size ~ 4 mm, Kimmel et al., 1995). Larvae at six days post fertilization were placed one per well in a 48-well plate (Falcon) in 1 mL of E3 medium. After an hour of acclimation to the plate, the plate was placed in a small chamber with recirculating water from a water bath set at 28.5 ºC. Each of the wells on the 48-well plate was designated as an individual ROI (Figure 3*A*). Video was acquired using an iPhone, and baseline motion of the larvae was recorded for 20 minutes. Wells in the dish were then partitioned into two groups. Larvae in odd-numbered wells received a dose of tricaine anesthetic (40 µL of 4 mg/mL; C_f: 160 µg/mL), while an equal volume of water was added to larvae in even-numbered wells. Tricaine-treated larvae exhibited a striking decrease in locomotion compared to their untreated siblings (Figure 3*B*, *C*). The water source for the recirculating bath was then switched to a reservoir at 46 ºC. Water temperature in wells reached maximum of 36 ºC after 10 minutes, as measured by a handheld thermometer. After 15 minutes of warm water treatment, the source for the recirculating bath was returned to 28.5 ºC. Untreated larvae exhibited a robust increase in locomotion, whereas their tricaine-treated siblings did not respond to the change in temperature (Figure 3*B*, *C*). Thus, video acquired from a mobile device was sufficient to quantify differences in motion of numerous microscopic animals simultaneously.

Long-term quantification of live video. To test the ability of our software to analyze a live video feed, to record data over an extended time period, and to quantify locomotion in real time, we performed a simple test of food preference in wild birds. A Logitech webcam (model C920) was placed on tripod and connected to an iMac computer, and the video stream was focused upon four small (12.5 cm x 8.5 cm) trays containing various kinds of bird food (from Pennington Ultra Nut & Fruit Blend), including peanuts, raisins, sunflower seeds, and miscellaneous small seeds (< 15 mm on longest axis, Figure 4*A*). The camera began recording at 9 am, and motion was recorded for a total of 27 hours. The ability of the camera to detect instances of feeding was confirmed via a live display of the pixel differences on the computer screen and simultaneous observations of visiting birds (Figure 4*E*, *F*). A variety of species were observed visiting the trays, including black-capped chickadees, house finches, and a pair of northern cardinals (Figure 4*E*, *F*). Significant motion was detected at all four trays during daylight hours (Figure 4*B*). Differences in feeding preference, however, were observable on a scale of hours and minutes (Figure 4*C*). Over the course of the experiment, the most motion was detected at the tray containing the small seeds, an intermediate amount of motion was detected at the trays containing sunflower seeds and peanuts, and the smallest amount of motion was detected at the tray containing the raisins (Figure 4*D*). These trends were recapitulated in an additional experiment of comparable length, in which the

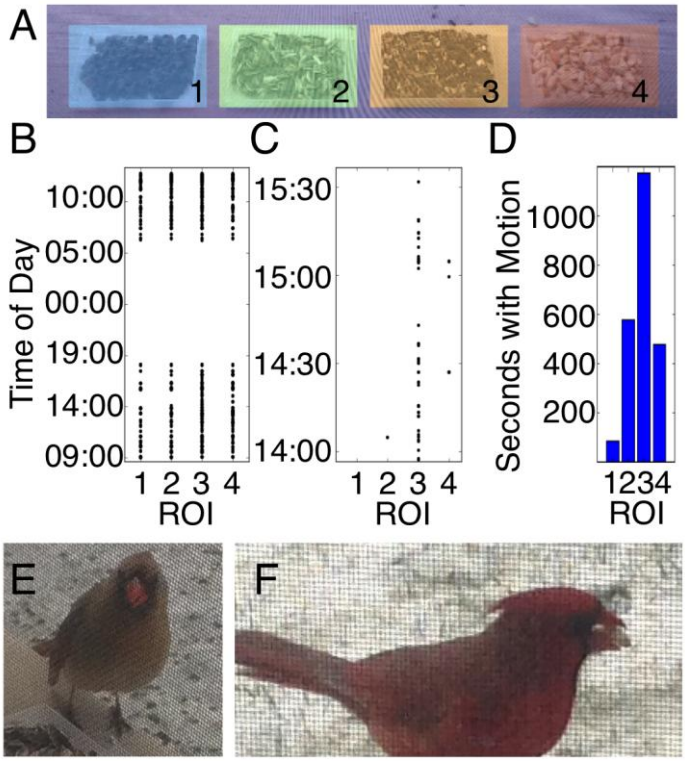

Figure 4. Long-term quantification of movement via a live webcam feed. *A.* Regions of interest defined in an image taken with a Logitech C920 webcam. The camera was situated behind a window with a screen mesh, and was pointed down at the exterior windowsill. ROI 1 = raisins, ROI 2 = sunflower seeds, ROI 3 = miscellaneous small seeds, ROI 4 = peanuts. *B-C.* Timing of movement recorded in each ROI. Frames with motion were represented by a dot at the appropriate time. All four trays of food were visited regularly during daylight hours (*B*). Between 14:00 (2pm) and 15:30 (3:30pm), significant motion was recorded in ROI 3. *D.* Quantification of movement in each ROI over entire experiment. *E-F.* Examples of birds enjoying a treat provided during the recording session.

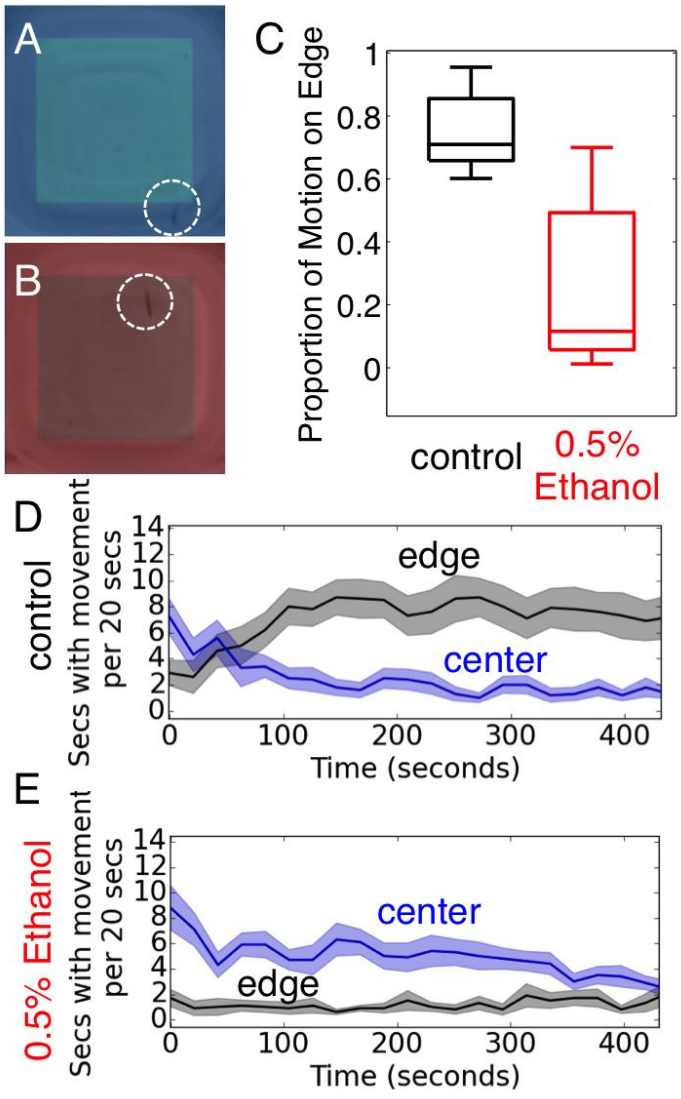

Figure 5. Ethanol decreases thigmotaxis behaviors in adult zebrafish. *A-B.* Frames taken from a movie recorded by a Panasonic HX-WA03 video camera, showing adult zebrafish in individual enclosures, filmed from above. ROIs were divided into inner and outer regions of equal area. Because of the rounded corners of the enclosures, the navigable outer region is smaller than the inner square. The white circles denote fish detected in the outer region (*A*), and the inner region (*B*) of the enclosures. *C.* Fish treated for one hour with 0.5% (vol/vol) ethanol exhibited less time in outer regions of their enclosures than do their control siblings ($p < 0.0001$ by two-tailed t-test; control: n=10; ethanol: n=10). *D.* Control fish exhibited a preference for the edge regions (black) of their enclosures. *E.* Ethanol-treated fish moved mostly in the center regions (blue). Plots are mean (dark line) +/- s.e.m. (shaded region), n=10 for each.

order of tray positioning was scrambled (not shown). Thus, our software is capable of long-term quantification of locomotor behavior, and can record and analyze behaviors in a live video feed. Therefore, our system enables analysis of behavioral patterns that emerge over extended periods of time, such as circadian behaviors or differences in locomotion that arise from long-term exposure to pharmacological agents.

Thigmotaxis behavior in adult zebrafish. To test the ability of our software to perform more complicated analyses of locomotion, we analyzed thigmotaxis behaviors in adult zebrafish exposed to a novel environment. Thigmotaxis, also known as wall-hugging or edge-seeking behavior, is the tendency of an animal to avoid the center region of a novel enclosure, and to exhibit preference for the boundary regions. Increased thigmotaxis has been associated with anxiety-like behaviors in a variety of species, including zebrafish (Champagne et al., 2010). We placed individual adult zebrafish into containers arrayed in a grid, and specified edge and center ROIs in each container with roiSelect.py (Figure 5 *A*, *B*). Untreated control fish exhibited a strong preference for the edge region of the enclosure during the seven-minute video (Figure 5*D*).

We then investigated the effects of acute ethanol exposure on thigmotaxis behavior. Prior to video recording, siblings of the control fish were treated for one hour with ethanol at a concentration of 0.5% (vol/vol). In previous work, this dose of ethanol decreased the response of fish to a predator-like stimulus, suggesting that ethanol treatment may be anxiolytic in adult fish (Gerlai et al., 2006). Here, the ethanol-treated fish, unlike their untreated siblings, lacked the strong preference for the container boundaries, and instead moved most often in the center region of their enclosures (Figure 5*E*). Thus, ethanol-treated fish exhibited a significant reduction in thigmotaxis (Figure 5*C*); the overall amount of movement, however, was not significantly different between ethanol-treated and control fish (not shown).

Automated video tracking enables objective, robust, and reproducible quantification of animal behaviors. Video-based quantification of behavior can provide significant advantages over manual data collection. For example, manual observations often require extensive training, and can be subject to experimenter bias and observer fatigue. In addition, the presence of an experimenter in the same room as the animals being studied can induce changes in the way animals behave (Sorge et al., 2014). The video tracking system we have developed here is flexible in terms of animals and experimental settings. Users can define regions of interest that are amenable to tracking large animals through different parts of their enclosures, quantifying place or feeding preferences in an open environment, and detecting motion simultaneously in animals arrayed in multi-well plates. Similarly, our software is flexible in terms of video input, and is able to analyze both live and recorded video from a variety of sources, including smartphone cameras, inexpensive webcams, and small handheld video cameras. The open source programming tools that are the foundation of our software are cross-platform and can be run on any personal computer. Furthermore, the software can be controlled via an optional Graphical User Interface (GUI), facilitating ease of use. Thus, the system we present here enables sophisticating video tracking analysis using inexpensive and nearly ubiquitous image capture devices and computer platforms. Moreover, because the software is free and open source, it is easily extendable, scalable, and

customizable to a variety of research questions and educational settings.

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Address correspondence to: Ian G. Woods, Dept. of Biology, 953 Danby Road, CNS 160, Ithaca, NY 14850. Email: iwoods@ithaca.edu